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Urine From Chronic Hepatitis B Virus Carriers: Implications for Infectivity

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Horizontal transmission of hepatitis B virus (HBV) without apparent sexual or parenteral exposure is common in hyperendemic areas. In most cases, the route of transmission is unknown. To investigate urine as a potential source of infection, serum and urine from 56 chronic hepatitis B surface antigen (HBsAg) carriers were examined for the presence of HBV DNA using the polymerase chain reaction (PCR). Thirty-four of the patients were anti-hepatitis B e antigen (anti-HBe) positive and 22 were hepatitis B e antigen (HBeAg) positive. HBV DNA was detected in serum from 46 patients (82%) and in urine from 28 patients (50%). Most HBeAg-positive patients had HBV DNA detectable in urine (91%), whereas urine samples from anti-HBe-positive patients were found to contain HBV DNA to a lesser extent (24%). When comparing HBV DNA from serum and urine by an end-point titration PCR, a titration difference averaging 10^3 was found between serum and urine. A significant female predominance was also noted among the positive urine samples ($P < 0.05$), which was not correlated to the presence of haematuria. Detection of HBV DNA may indicate active viral replication, and thereby infectivity. Because a high proportion of chronic HBV carriers were found to have HBV DNA in urine, it is suggested that irrespective of HBeAg/anti-HBe status, urine should be regarded as a potential route of transmission and therefore be investigated further as a means of horizontal and nosocomial transmission of HBV. ***J. Med. Virol. 60: 17–20, 2000.***

KEY WORDS: HBV DNA; polymerase chain reaction; transmission; HBeAg; anti-HBe

INTRODUCTION

Vertical, sexual, and parenteral transmission of hepatitis B virus (HBV) has been well documented. Among very small and pre-adolescent children, however, horizontal transmission without apparent parenteral exposure is a common mode of acquisition [Davis et al., 1989; Martinson et al., 1998]. This route applies especially to high endemicity settings. In areas of low HBV endemicity, horizontal transmission may explain secondary cases of HBV among households and day-care centres that have a persistent carrier [Davis et al., 1989].

Hepatitis B surface antigen (HBsAg) has been detected in most body fluids and secretions, such as saliva, semen, breast milk, vaginal secretions, urine, and pancreatic and biliary secretions [Boxall et al., 1974; Darani and Gerber, 1974; Heathcote et al., 1974; Irwin et al., 1975; Hoefs et al., 1980; Badur et al., 1992]. The presence of HBsAg in faeces, however, is more controversial [Grabow et al., 1975; Irwin et al., 1975]. HBsAg is not an optimal infectivity indicator, however, because it may be found in the absence of circulating virions. Detection of HBV DNA in serum indicates active viral replication, and is therefore a more relevant infectivity marker. HBV will not infect standard cell cultures, and has a very narrow host range. The infectivity of HBV preparations can thus be tested only by inoculation of chimpanzees and a few other primates. Ulrich et al. [1989] found that polymerase chain reaction (PCR) was more sensitive than in vivo infectivity titration of HBV by inoculation of chimpanzees. Based on these experiments, they suggested that specimens in which no HBV DNA can be amplified by PCR probably do not contain infectious HBV. However, this conclusion can be drawn only after the sensitivity and specificity of a PCR assay has been determined.

Studies using molecular hybridisation techniques have shown HBV DNA to be present in semen, saliva, and urine [Karayiannis et al., 1985; Davison et al., 1987]. According to these studies, 55–60% of hepatitis B e antigen (HBeAg)-positive chronic carriers were

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TABLE I. Number of Chronic HBV Carriers Who Were HBV DNA Positive in Serum and Urine, According to Gender and HBeAg/anti-HBe Status

	HBeAg-positive		anti-HBe positive		
	Females (n = 11)	Males (n = 11)	Females (n = 13)	Males (n = 21)	Total
HBV DNA positive in serum	11	11	8	16	46/56 (82%)
HBV DNA positive in urine	11	9	5	3	28/56 (50%)

HBV, hepatitis B virus; HBeAg, hepatitis B e antigen.

HBV DNA positive in urine. To our knowledge, no extensive study looking at the presence of HBV DNA in the urine of chronic carriers (including a cohort of anti-HBe-positive patients) using PCR has been performed. The aim of this study was therefore to determine the proportion of chronic carriers who have HBV DNA, detectable by PCR, in urine. We also wanted to compare the levels of HBV DNA in serum and urine by a semi-quantitative PCR.

Traditionally, only sexual and bloodborne transmission of HBV has been indicated. If a large number of patients excrete HBV DNA in their urine, this would be an additional source of infection and must also be considered seriously, particularly for the care of infants and young children, and also in the context of nosocomial infections.

MATERIAL AND METHODS

Patients and Samples

Serum and urine were collected from 56 chronic HBsAg carriers attending the Department of Infectious Diseases, University Hospital of Lund. There were 32 men and 24 women, with a median age of 24 years. Thirty-four patients were anti-HBe positive and 22 were HBeAg positive. None of the patients had any known renal disease or had received antiviral treatment. The HBV genotypes had been determined from 32 of the 56 carriers by standard phylogenetic analysis of amplified sequences [Kidd-Ljunggren et al., 1995a, 1995b]. Most patients carried genotype D (16 patients), whereas the remaining had genotypes A (5 patients), B (1 patient), C (7 patients), and E (3 patients).

Samples of serum and urine were collected on the same day and stored at −20°C. Sequential serum and urine samples were collected from 12 patients. HBV markers in serum were tested for with commercial test kits (Abbott Laboratories, North Chicago, IL). Specimens of urine were tested for the presence of blood with Hemastix (Bayer Corporation, USA).

The serum alanine aminotransferase (ALT) value was available from a number of patients, measured on an aliquot of the sample that was tested by PCR. The upper normal level in our clinical chemistry laboratory is 0.7 ukat/L.

PCR

DNA was extracted from serum and urine by the phenol-chloroform method, and amplified as described previously [Ljunggren and Kidd, 1991] with minor modifications in cycling temperatures (94°, 45°, 72°) and using primer pairs KL1 (5′ GCT TTG/A GGG CAT GGA CAT TGA CCC GTA TAA 3′) and 2032R [Kaneko et al., 1989]; and KL28 (5′ GAG ACC ACC GTG AAC GCC 3′) and KL6 (5′ GGA AAG AAG TCA GAA GGC A 3′), respectively. The sensitivity of the PCR using primer pair KL1 and 2032R has been reported previously [Ljunggren and Kidd, 1991] and we have recently confirmed the detection level to be 1–10 genome copies. Primer pair KL 28 and KL6 is marginally less sensitive, but allows subsequent sequencing of the core promotor and precore regions. The amplified DNA was run on 1.8% agarose gels with ethidium bromide and visualised by ultraviolet light.

Great care was taken throughout the procedure to avoid DNA carryover [Kwok and Higuchi, 1989]. Disposable plastic pipettes and positive displacement pipettors (Microman; Gilson, France) were used during the whole procedure. There was never any evidence of DNA contamination in the controls with no DNA.

Correlation Between HBV DNA in Serum and Urine

To compare the levels of HBV DNA in serum and urine, an end-point titration PCR down to 10^{-6} was made from extracted serum and urine from 10 patients (9 HBeAg positive and 1 anti-HBe positive). The extracted material was diluted in sterile double-distilled water in log steps before PCR testing.

Statistical Method

The Chi square test was used for statistical analysis.

RESULTS

HBV DNA was detected in serum from 46 of 56 HBsAg-positive patients (Table I). Serum samples from all 22 HBeAg-positive patients and from 24 of the 34 anti-HBe-positive patients were HBV DNA positive. Urine samples from 28 of the 46 patients with HBV DNA in their serum were HBV DNA positive (61%). Twenty of these patients were HBeAg positive. All patients with HBV DNA in urine also had positive results in the corresponding serum samples. There was no correlation between HBV genotype and PCR positivity in urine.

More than one sample pair was available from five patients who were HBV DNA positive in urine. The urine from these patients was consistently positive for HBV DNA. From one patient, all serum and urine specimens collected on 13 different occasions over 6 years were PCR positive.

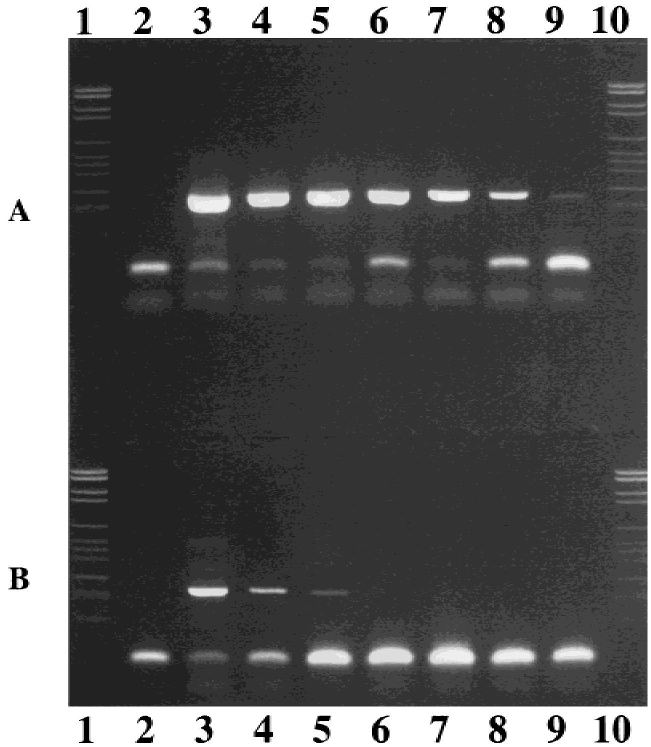

Fig. 1. Serially diluted hepatitis B virus DNA detected by polymerase chain reaction (PCR) using primer pair KL1 and 2032R from the serum and urine of a chronic hepatitis B surface antigen (HBsAg) carrier. **A:** serum; **B:** urine. **Lanes 1 and 10:** Molecular weight marker (a mixture of fragments from cleavage of pBR328 DNA with *Bg1* I and *Hinf* I). **Lane 2:** Negative control with water instead of DNA. **Lane 3:** Undiluted patient DNA. **Lanes 4–9:** Patient DNA diluted 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6}, respectively.

One HBV DNA-positive urine sample contained detectable but minute amounts of blood. Conversely, one urine sample with microscopic haematuria was HBV DNA negative, although the corresponding serum sample was HBV DNA positive.

ALT levels were available from 18 of the patients with HBV DNA detectable in urine. Nine were within the normal range (< 0.7 ukat/L) and nine had elevated levels.

In the PCR with end-point titration of extracted HBV DNA from 10 patients, the serum samples were positive to a dilution of 10^{-3} to 10^{-6}, whereas the urine samples were positive to a dilution of 10^{-1} to 10^{-3} (Fig. 1). The difference in titre between serum and urine ranged from 10 to 10^4. This difference in concentration is illustrated by comparing serum and urine from different carriers (Fig. 2).

There was a significant predominance of female patients among those with HBV DNA-positive urine specimens. Eleven of the 20 HBeAg-positive patients with detectable HBV DNA in urine and five of the eight anti-HBe-positive patients with HBV DNA in urine were female (Table I). Thus, HBV DNA was found in the urine of 67% of the women, compared with 37% of the men ($P < 0.05$).

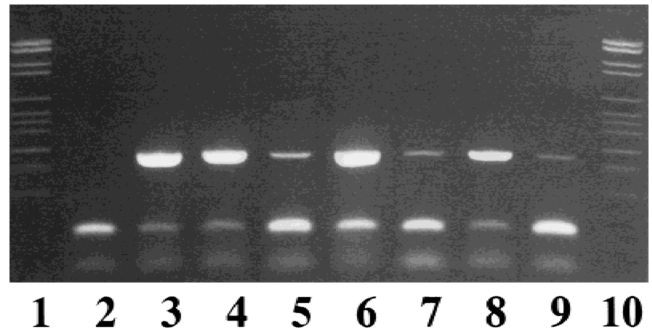

Fig. 2. Comparison of the polymerase chain reaction (PCR) results from the serum and urine of 3 chronic hepatitis B surface antigen (HBsAg) carriers, using primer pair KL1 and 2032R. **Lanes 1 and 10:** Molecular weight marker (a mixture of fragments from cleavage of pBR328 DNA with *Bg1* I and *Hinf* I). **Lane 2:** Negative control with water instead of DNA. **Lane 3:** Positive control. **Lanes 4–9:** Serum and urine DNA from three patients, arranged in pairs (serum and urine).

DISCUSSION

The aim of this study was to investigate the presence of HBV DNA in urine from chronic HBsAg carriers. The detection of HBV DNA in urine may indicate the presence of virus particles and hence that urine is a potential source of infection. Using PCR, HBV DNA was detected in the urine of 50% of the patients. The presence of HBV DNA was considerably higher in urine from HBeAg-positive patients. HBV DNA was also found in serum from most anti-HBe-positive patients, and to a lesser extent also in urine.

The results of end-point titration indicate that detectable levels of virus in urine are approximately 10^3-fold less than those found simultaneously in serum. The same relationship has also been observed between levels of HBV DNA in serum and other body fluids, such as saliva and semen [Jenison et al., 1987].

Negative tests for erythrocytes in most urine specimens ruled out blood contamination as a major cause of the urinary HBV DNA. Yet there is still a possibility that an altered glomerular permeability could account for the HBV DNA detectable in some patients. Another plausible way for virions to reach the urinary tract, besides the possible “leakage” of free viral particles into the urine, is by means of leukocytes and to a minor extent shed renal epithelial cells, where HBV DNA has been demonstrated as well as in hepatocytes [Bouffard et al., 1990; He et al., 1998]. In this way, a larger amount of HBV DNA would be expected during times of increased local epithelial and white cell count, such as during disease or inflammatory reactions in the urinary tract. However, five patients who were HBV DNA positive in urine and from whom there was more than one sample available, were continuously positive for HBV DNA in urine. These findings do not support the theory that HBV DNA is excreted only during illness. The presence of epithelial cells and leukocytes in urine is normally higher in women [Prinz et al., 1993], which may explain the higher rate of female patients with HBV DNA in their urine found in this study.

Degradative enzymes produced by bacteria are an additional factor that may influence the detection of HBV DNA in urine. This applies to certain *Pseudomonas* species, which have been shown to produce an HBV antagonist [Grabow et al., 1975]. Urine is normally sterile, but instrumentation may lead to iatrogenic bacteriuria. None of our patients had been subjected to any kind of invasive procedures in the genitourinary tract before sampling.

A number of studies emphasise that horizontal transmission without apparent sexual or parenteral exposure is an important route of HBV infection in hyperendemic areas, especially during early childhood [Davis et al., 1989; Martinson et al., 1998]. These infections are acquired most likely within households in which a persistent carrier is present. Infected saliva and minor wounds are believed to be responsible for some of these transmissions, even though the causes of most of them remain uncertain. The results of this study indicate that urine should also be considered as a potential source of HBV infection.

It is concluded that a high proportion of patients chronically infected with HBV have HBV DNA detectable by PCR in their urine. The results indicate that urine from all HBsAg carriers could harbour infectious HBV, and not just urine from HBeAg-positive patients. These findings might explain the horizontal transmission of HBV among small children, and must be considered seriously in matters of hospital hygiene and the risk of nosocomial transmission.

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